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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/705 — A1

(11) International Publication Number: WO 98/25967

(43) International Publication Date: 18 June 1998 (18.06.98)

(21) International Application Number: PCT/US97/22278

(22) International Filing Date: 5 December 1997 (05.12.97)

(30) Priority Data:
60/032,705 12 December 1996 (12.12.96) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HVEM POLYPEPTIDES AND USES THEREOF

(57) Abstract

Novel polypeptides, designated HVEM, are provided. Compositions including HVEM chimeras, nucleic acid encoding HVEM, and antibodies to HVEM are also provided.

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HVEM Polypeptides and Uses Thereof

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of polypeptides, designated herein as herpesvirus entry mediator ("HVEM") and to uses thereof.

BACKGROUND OF THE INVENTION

Various molecules, such as tumor necrosis factor-α ("TNF-α"), tumor necrosis factor-β ("TNF-β" or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), TRAIL, and Apo-2 ligand have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)].

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH_2-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., Cell, 66:233-243 (1991)]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and

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myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF-α or anti-Apo-1 mouse monoclonal antibody [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin-α, are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF-α, Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), play a role in inactivation of mature lymphocytes by triggering apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell; 85:781-784 (1996); Nagata et al., Science, 267:1449-1456 (1995); Zheng et al., Nature, 377:348-351 (1995)]. TNFR1 is also known to mediate activation of the transcription factor, NF-κB [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

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Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the thiol protease MACHα/FLICE into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACHα/FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 β converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-κB [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF-κB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-κB is complexed with members of the IκB inhibitor family; upon inactivation of the IκB in response to certain stimuli, released NF-κB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription. TNFR proteins may also regulate the AP-1 transcription factor family [Karin, J. Biol. Chem., 270:16483-16486 (1995)]. AP-1 represents a separate family of dimeric transcriptional activators composed of members of the Fos and Jun protein families [Karin, supra]. AP-1 activation is believed to be mediated by immediate-early induction of *fos* and *jun* through the mitogen-activated protein kinases ERK and JNK, as well as by JNK-dependent phosphorylation of Jun proteins [Karin, supra]. Transcriptional regulation by TNFR family members is mediated primarily by members of the TNF receptor associated factor (TRAF) family [Rothe et al., Cell, 78:681-692 (1994); Hsu et al., Cell, 84:299-308 (1996); Liu et al., Cell, 87:565-576 (1996)].

Montgomery et al. recently identified a member of the TNFR family, called "HVEM" [Montgomery et al., Cell, 87:427-436 (1996)]. According to Montgomery et al., the HVEM mediated efficient entry of HSV-1 strains into CHO-K1 cells and ST cells and enhanced the entry of an HSV-2 strain. The nucleotide sequence of the HVEM cDNA and amino acid sequence of the open reading frame is described in Figure 2 of the Montgomery et al. reference.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode polypeptides, designated in the present application as "HVEM." It is believed that HVEM is a member of the TNFR family. Applicants found that transient transfection of HVEM into human 293 cells caused marked activation of certain transcription factors, suggesting that HVEM is involved in regulating gene expression in response to infectious stimuli and cellular stress. The predominant expression of HVEM mRNA in lymphocyte-rich tissues such as spleen and peripheral blood also suggests its role as a receptor in regulating lymphocyte activity.

In one embodiment, the invention provides isolated HVEM polypeptide. In particular, the invention provides isolated native sequence HVEM polypeptide, which in one embodiment, includes an amino

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acid sequence comprising residues 1 to 283 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated HVEM polypeptide comprises at least about 80% identity with native sequence HVEM polypeptide comprising residues 1 to 283 of Figure 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising HVEM polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an HVEM fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of HVEM fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding HVEM polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an HVEM polypeptide or a particular domain of HVEM, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderately stringent conditions and optionally, under stringent conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 283, inclusive; or

(b) a sequence corresponding to the sequence of (a) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the HVEM polypeptide or particular domain of HVEM. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing HVEM is further provided.

In another embodiment, the invention provides an antibody which specifically binds to HVEM. The antibody may be an agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include HVEM or HVEM antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of HVEM cDNA (SEQ ID NO:2) and its derived amino acid sequence (SEQ ID NO:1).

Figure 2 shows activation of NF-κB by ectopic expression of HVEM.

Figure 3 shows activation of AP-1 by ectopic expression of HVEM.

Figure 4 illustrates expression of HVEM mRNA in human tissues as determined by Northern blot hybridization.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "herpesvirus entry mediator" and "HVEM" when used herein encompass native sequence HVEM and HVEM variants (each of which is defined herein). These terms encompass HVEM from a variety of mammals, including humans. The HVEM may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

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A "native sequence HVEM" comprises a polypeptide having the same amino acid sequence as an HVEM derived from nature. Thus, a native sequence HVEM can have the amino acid sequence of naturally-occurring HVEM from any mammal. Such native sequence HVEM can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence HVEM" specifically encompasses naturally-occurring truncated or secreted forms of the HVEM (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants of the HVEM. A naturally-occurring variant form of the HVEM includes an HVEM having at codon 108, a serine or a threonine, and at codon 140, an alanine or an arginine, according to the sequence numbering shown in Figure 1. In one embodiment of the invention, the native sequence HVEM is a mature or full-length native sequence HVEM comprising the amino acid sequence of Figure 1 (SEQ ID NO:1). The present definition of native sequence HVEM excludes known EST sequences, such as GenBank AA021617.

"HVEM variant" means a biologically active HVEM having less than 100% sequence identity with HVEM having the deduced amino acid sequence shown in Figure 1 (SEQ ID NO:1) for a full-length native sequence HVEM. Such HVEM variants include, for instance, HVEM polypeptides wherein one or more amino acid residues are added at the N- or C-terminus of, or within, the sequence of Figure 1; from about one to 30 amino acid residues are deleted, or optionally substituted by one or more amino acid residues; and derivatives thereof, wherein an amino acid residue has been covalently modified so that the resulting product has a non-naturally occurring amino acid. Ordinarily, an HVEM variant will have at least about 80% sequence identity, more preferably at least about 90% sequence identity, and even more preferably at least about 95% sequence identity with the sequence of Figure 1 (SEQ ID NO:1). The present definition of HVEM variant excludes known EST sequences, such as GenBank AA021617.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising HVEM, or a portion thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the HVEM. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the HVEM natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

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An "isolated" HVEM nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the HVEM nucleic acid. An isolated HVEM nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated HVEM nucleic acid molecules therefore are distinguished from the HVEM nucleic acid molecule as it exists in natural cells. However, an isolated HVEM nucleic acid molecule includes HVEM nucleic acid molecules contained in cells that ordinarily express HVEM where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-HVEM monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-HVEM antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-HVEM antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production

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of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated HVEM polypeptides. In one embodiment, the HVEM comprises the amino acid sequence shown in Figure 1 (SEQ ID NO:1). Optionally, in the extracellular region, codon 108 (using the sequence numbering of Figure 1) encodes a serine or threonine and codon 140 encodes an alanine or arginine. Typically, the HVEM does not include a death domain in the cytoplasmic region of the polypeptide.

The properties and characteristics of HVEM polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the HVEM polypeptides disclosed herein, it is Applicants' present belief that HVEM is a member of the TNFR family.

A description follows as to how HVEM, as well as HVEM chimeric molecules and anti-HVEM antibodies, may be prepared.

A. Preparation of HVEM

The description below relates primarily to production of HVEM by culturing cells transformed or transfected with a vector containing HVEM nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare HVEM.

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1. Isolation of DNA Encoding HVEM

The DNA encoding HVEM may be obtained from any cDNA library prepared from tissue believed to possess the HVEM mRNA and to express it at a detectable level. Accordingly, human HVEM DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human retinal cDNA described in Example 1. The HVEM-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the HVEM or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding HVEM is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library with an oligonucleotide probe. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P-labeled ATP, biotinylation or enzyme labeling.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

HVEM variants can be prepared by introducing appropriate nucleotide changes into the HVEM DNA, or by synthesis of the desired HVEM polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the HVEM, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native sequence HVEM as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis.

2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding HVEM may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

(i) Signal Sequence Component

The HVEM may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a

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specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the HVEM DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native HVEM presequence that normally directs insertion of HVEM in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding HVEM.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of HVEM DNA. However, the recovery of genomic DNA encoding HVEM is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the HVEM DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not

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survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the HVEM nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes HVEM. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of HVEM are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding HVEM. This amplification technique can be used with any otherwise suitable host, *e.g.*, ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding HVEM, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence

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of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20.622 or 38.626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the HVEM nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the HVEM nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to HVEM encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native HVEM promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the HVEM DNA.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding HVEM [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding HVEM.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase,

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glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

HVEM transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the HVEM sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the HVEM of this invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples

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include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the HVEM coding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding HVEM.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding HVEM may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying HVEM variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of HVEM in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B.*

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licheniformis (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for HVEM-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated HVEM are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, *e.g.*, Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the HVEM can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the HVEM-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, *e.g.*, Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

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Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for HVEM production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

4. Culturing the Host Cells

Prokaryotic cells used to produce HVEM may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce HVEM may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

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In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence HVEM polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to HVEM DNA and encoding a specific antibody epitope.

6. Purification of HVEM Polypeptide

Forms of HVEM may be recovered from culture medium or from host cell lysates. If the HVEM is membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When HVEM is produced in a recombinant cell other than one of human origin, the HVEM is free of proteins or polypeptides of human origin. However, it may be desired to purify HVEM from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to HVEM. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. HVEM thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as

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DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

HVEM variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence HVEM, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of an HVEM fusion with another protein or polypeptide, *e.g.*, a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence HVEM may require modification to account for changes in the character of HVEM or its variants upon expression in recombinant cell culture.

7. Covalent Modifications of HVEM Polypeptides

Covalent modifications of HVEM are included within the scope of this invention. One type of covalent modification of the HVEM is introduced into the molecule by reacting targeted amino acid residues of the HVEM with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the HVEM.

Derivatization with bifunctional agents is useful for crosslinking HVEM to a water-insoluble support matrix or surface for use in the method for purifying anti-HVEM antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking HVEM molecules to generate HVEM dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the HVEM polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties

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found in native sequence HVEM, and/or adding one or more glycosylation sites that are not present in the native sequence HVEM.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the HVEM polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence HVEM (for O-linked glycosylation sites). The HVEM amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the HVEM polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the HVEM polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the HVEM polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duksin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of HVEM comprises linking the HVEM polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or

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polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

8. HVEM Chimeras

The present invention also provides chimeric molecules comprising HVEM fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the HVEM with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the HVEM. The presence of such epitope-tagged forms of the HVEM can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the HVEM to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α-tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

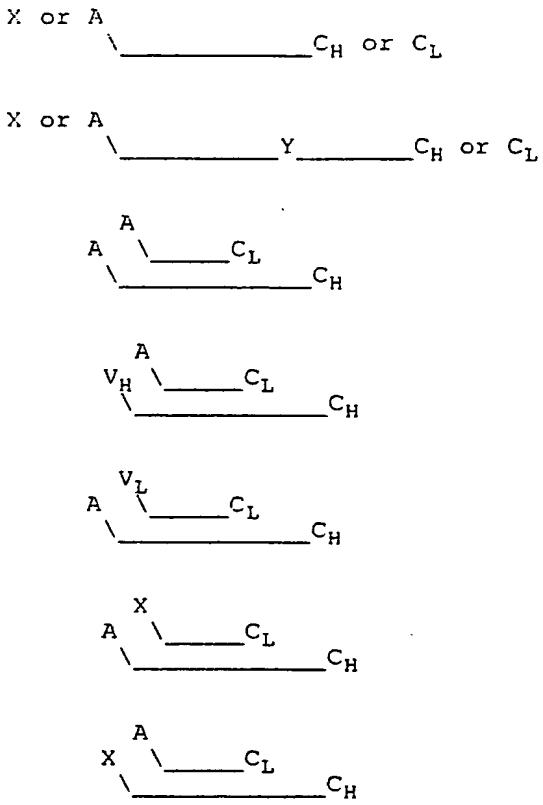
Generally, epitope-tagged HVEM may be constructed and produced according to the methods described above. HVEM-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the HVEM portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the HVEM-tag polypeptide chimeras of the present invention, nucleic acid encoding the HVEM will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N- terminus or the C- terminus and used as a purification handle in affinity chromatography.

Epitope-tagged HVEM can be purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged HVEM can then be eluted from the affinity column using techniques known in the art.

In another embodiment, the chimeric molecule comprises an HVEM polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of HVEM, such as the extracellular domain sequence of native HVEM fused to an immunoglobulin sequence. This includes chimeras in monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms.

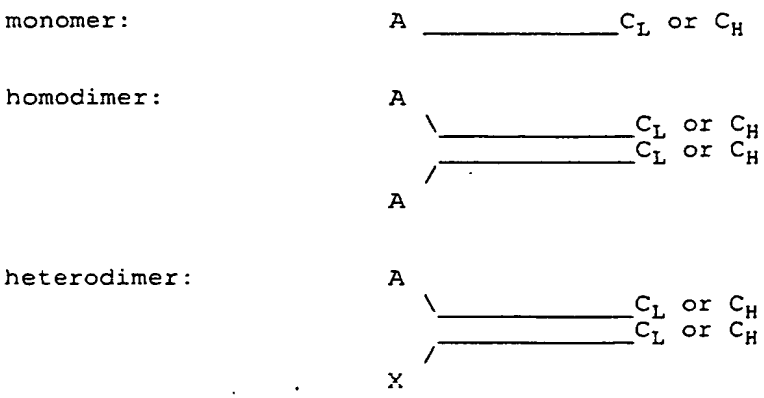
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Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.



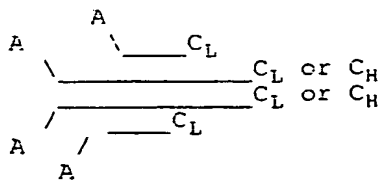
A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

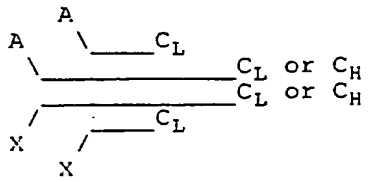


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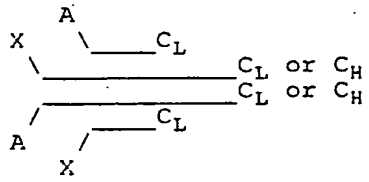
homotetramer:



heterotetramer:



and



In the foregoing diagrams, "A" means an HVEM sequence or an HVEM sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon-γ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and V_L, V_H, C_L and C_H represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an HVEM sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed as follows. The DNA including a region encoding the desired sequence, such as an HVEM and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point

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at or near the DNA encoding the N-terminal end of the HVEM or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci., USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989. Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Uses for HVEM

Nucleic acid sequences encoding the HVEM may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding HVEM is present in the cell type(s) being evaluated. HVEM nucleic acid will also be useful for the preparation of HVEM by the recombinant techniques described herein.

The isolated HVEM may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of HVEM may be prepared. HVEM preparations are also useful in generating antibodies, as standards in assays for HVEM (*e.g.*, by labeling HVEM for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the HVEM, such as the HVEM-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-HVEM antibodies. Such modified forms of the HVEM may also be employed as inhibitors of HSV infection and/or of native ligands of HVEM.

Nucleic acids which encode HVEM or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an

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embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding HVEM or an appropriate sequence thereof can be used to clone genomic DNA encoding HVEM in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding HVEM. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for HVEM transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding HVEM introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding HVEM. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with HSV infection. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of HVEM such as the HVEM ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of the ligand of HVEM. Such animals could also be challenged, for example, by infectious agents like HSV or *E. coli* and tested for susceptibility to infection or its consequences.

Alternatively, non-human homologues of HVEM can be used to construct a HVEM "knock out" animal which has a defective or altered gene encoding HVEM as a result of homologous recombination between the endogenous gene encoding HVEM and altered genomic DNA encoding HVEM introduced into an embryonic cell of the animal. For example, cDNA encoding HVEM can be used to clone genomic DNA encoding HVEM in accordance with established techniques. A portion of the genomic DNA encoding HVEM can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the HVEM polypeptide.

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C. Anti-HVEM Antibody Preparation

The present invention further provides anti-HVEM antibodies. Antibodies against HVEM may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The HVEM antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the HVEM polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a HVEM-IgG fusion protein. Cells expressing HVEM at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

2. Monoclonal Antibodies

The HVEM antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the HVEM polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a HVEM-IgG fusion protein or chimeric molecule. Cells expressing HVEM at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase

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(HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against HVEM. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

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The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH_1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH_1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

3. Humanized Antibodies

The HVEM antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from

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an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of

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Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the HVEM, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for

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treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

D. Uses for HVEM Antibodies

The HVEM antibodies of the invention have therapeutic utility. Antagonistic antibodies, for instance, may be used to block potential excessive inflammatory or autoimmune effects of HVEM activation resulting from NF-κB or AP-1 induction or to inhibit HSV infection. Agonistic HVEM antibodies may be used to supplant or supplement activity of the HVEM ligand as it pertains to regulating NF-κB or AP-1-induced gene expression.

HVEM antibodies may further be used in diagnostic assays for HVEM, *e.g.*, detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

HVEM antibodies also are useful for the affinity purification of HVEM from recombinant cell culture or natural sources. In this process, the antibodies against HVEM are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the HVEM to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the HVEM, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the HVEM from the antibody.

E. Kits Containing HVEM or HVEM Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing HVEM or HVEM antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is HVEM or an HVEM antibody. The label on the container indicates that the composition is used

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for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

EXAMPLE 1

Isolation of cDNA clones Encoding HVEM

To isolate a cDNA for HVEM, a bacteriophage library of human retinal cDNA (commercially available from Clontech) was screened by hybridization with a synthetic oligonucleotide probe based on an EST sequence (GenBank locus AA021617), which showed some degree of homology to members of the TNFR family. The oligonucleotide probe employed in the screening was 60 bp long. Hybridization was conducted with a 1:1 mixture overnight at room temperature in buffer containing 20% formamide, 5X SSC, 10% dextran sulfate, 0.1% $NaPiPO_4$, 0.05M $NaPO_4$, 0.05 mg salmon sperm DNA, and 0.1% sodium dodecyl sulfate, followed consecutively by one wash at room temperature in 6X SSC, two washes at 37°C in 1X SSC/0.1% SDS, two washes at 37°C in 0.5X SSC/0.1% SDS, and two washes at 37°C in 0.2X SSC/0.1% SDS. Five positive clones (containing cDNA inserts of 1.8-1.9kb) were identified in the cDNA library, and the positive clones were confirmed to be specific by PCR using the above hybridization probe as a PCR primer. Single phage plaques containing each of the five positive clones were isolated by limiting dilution and the DNA was purified using a Wizard Lambda Prep DNA purification kit (commercially available from Promega).

The cDNA inserts from three of the five bacteriophage clones were excised from the vector arms by digestion with EcoRI, gel-purified, and subcloned into pRK5 and sequenced on both strands. The three clones contained an identical open reading frame (with the exception of an intron found in one of the clones).

The entire nucleotide sequence of HVEM is shown in Figure 1. The cDNA contained one open reading frame with a translational initiation site assigned to the ATG codon at nucleotide positions 86-88. The surrounding sequence at this site is in reasonable agreement with the proposed consensus sequence for initiation sites [Kozak, J. Cell. Biol., 115:887-903 (1991)]. The open reading frame ends at the termination codon TGA at nucleotide positions 925-927.

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The predicted amino acid sequence of the full length HVEM contains 283 amino acids (See Fig. 1(SEQ ID NO:1)). A putative transmembrane region of the HVEM comprises amino acids 201-225 of Fig. 1 and a putative cytoplasmic region of the HVEM comprises amino acids 226-283 of Fig. 1. The sequence differs from the HVEM sequence reported in Montgomery et al., supra, in at least two amino acids: as shown in Fig. 1, codon 108 encodes a serine and codon 140 encodes an alanine. An alignment (using the AlignTM computer program) of a 58 amino acid long cytoplasmic region of HVEM with other known members of the human TNF receptor family showed some sequence similarity, in particular to CD40 (12 identities) and LT-beta receptor (11 identities).

EXAMPLE 2

Activation of NF-κB by HVEM

An assay was conducted to determine whether HVEM activates NF-κB.

HEK 293 cells (ATCC) were transiently transfected by the calcium precipitation method with pRK5 or with pRK5-based expression vectors for HVEM (see Example 1), TNFR1 or Apo-3 (Genentech, Inc.) (10 μg/10cm dish). Nuclear extracts were prepared 24 hours later as described by Marsters et al., Proc. Natl. Acad. Sci., 92:5401-5405 (1995). As shown in Fig. 2(a), aliquots from each extract (1 μg total protein) was reacted (as described in Marsters et al., supra) with a ^{32}P-labelled oligonucleotide based on a mutated NF-κB target sequence (control radioprobe; plus strand sequence 5'-AGTTGAGGCGACTTTCCCAGGC-3' (SEQ ID NO:3)) [see, also, Lenardo et al., Cell, 58:227-229 (1989)], or with a ^{32}P-labelled oligonucleotide based on a wild-type NF-κB target sequence (plus strand sequence 5'-AGTTGAGGGGACTTTCCCAGGC-3' (SEQ ID NO:4) [see, Lenardo et al., supra]. The NF-κB radioprobe was added alone or together with a 50-fold excess of unlabelled oligonucleotide of the same sequence (cold oligo). The reactions were subjected to electrophoresis on polyacrylamide gels and visualized by phosphorimager analysis (electrophoretic mobility shift assay "EMSA") as described in Marsters et al., supra. The position of the NF-κB- specific band is indicated by an arrow in Fig. 2a.

Aliquots from each nuclear extract were also incubated with the NF-κB specific radioprobe together with rabbit preimmune serum or with rabbit anti-p65/Rel A IgG (purchased from Santa Cruz Biotechnology) and analyzed by EMSA as above. The positions of the nonshifted and the antibody shifted NF-κB probe are indicated by arrows in Fig. 2.

The cells transfected by the pRK5-based HVEM expression plasmid showed significant NF-κB activation relative to cells transfected by pRK5 alone. The effect of transfection by TNFR1 or Apo-3/DR-3 was tested for comparison. TNFR1 and Apo-3/DR-3 have previously been shown to activate NF-κB [Tartaglia et al., Cell, 74:845-853 (1993); Chinnaiyan et al., Science, 274:990-992 (1996); Genentech, Inc. (data not shown)]. The level of NF-κB activation by these receptors was similar to the level of activation by HVEM. The specific antibody to the p65/RelA subunit of NF-κB, but not the preimmune serum, inhibited mobility of the NF-κB probe in the case of each receptor (Fig. 2b). Thus, the NF-κB complexes activated by HVEM, TNFR1 and Apo-3 in HEK293 cells contain the p65/RelA protein.

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EXAMPLE 3

Activation of AP-1 by HVEM

An assay was conducted to determine whether HVEM activates AP-1.

HEK 293 cells were transiently transfected with pRK5 or with pRK5-based expression vectors for HVEM (see Example 1) or TNFR1 (10 μg/10cm dish). Nuclear extracts were prepared 24 hours later as described above in Example 2. Aliquots from each extract (1 μg total protein) were reacted (as described in Marsters et al., supra) with a ^{32}P-labelled oligonucleotide based on a mutated AP-1 target sequence (control radioprobe; plus strand sequence 5'-CGCTTGATGACTTGGCCGGAA-3' (SEQ ID NO:5)) [see, also, Lee et al., Cell, 49:741-752 (1987)], or with a ^{32}P-labelled oligonucleotide based on a wild-type AP-1 target sequence (plus strand sequence 5'-CGCTTGATGACTCAGCCGGAA-3' (SEQ ID NO:6) [see, Lee et al., supra]. The AP-1 radioprobe was added alone or together with a 50-fold excess of unlabelled oligonucleotide of the same sequence (cold oligo). The reactions were analyzed as described in Example 2 above. The position of the AP-1- specific band is indicated by an arrow in Fig. 3a.

Aliquots from each nuclear extract were also incubated with the AP-1 specific radioprobe together with rabbit preimmune serum or with rabbit anti-Jun B, anti-c-Jun, or anti-Jun D IgG (purchased from Santa Cruz Biotechnology) and analyzed by EMSA as above. The positions of the nonshifted and the antibody shifted AP-1 probe are indicated by arrows in Fig. 3b.

The HVEM-transfected cells showed marked AP-1 activation as compared to cells transfected by pRK5 alone; the level of activation was similar to the level of activation by TNFR1. (Fig. 3a). Anti-Jun D antibody, but not antibody to c-Jun or Jun B or preimmune serum, inhibited the migration of the AP-1 specific probe (Fig. 3b), indicating that Jun D participated in the AP-1 complexes activated by HVEM and TNFR1 in HEK293 cells. Jun D was previously shown to be activated by TNF [Bierhaus et al., J. Biol. Chem., 270:26419-26432 (1995)].

EXAMPLE 4

Northern Blot Analysis

Expression of HVEM mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a NheI/SacI cDNA fragment containing the entire HVEM coding region (see Example 1). Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probe. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 4, a predominant mRNA transcript of approximately 1.8kb was detected in multiple adult (Fig. 4 a,b) and fetal (Fig. 4c) tissues. The predominant sites of expression were lymphocyte-rich tissues such as spleen and peripheral blood. A second transcript of about 3.8kb, as well as some larger transcripts also were detected.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: HVEM Polypeptides and Uses Thereof

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Marschang, Diane L.
(B) REGISTRATION NUMBER: 35,600
(C) REFERENCE/DOCKET NUMBER: P1068PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-5416
(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 283 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Glu Pro Pro Gly Asp Trp Gly Pro Pro Pro Trp Arg Ser Thr
 1               5                  10                  15

Pro Arg Thr Asp Val Leu Arg Leu Val Leu Tyr Leu Thr Phe Leu
            20                  25                  30

Gly Ala Pro Cys Tyr Ala Pro Ala Leu Pro Ser Cys Lys Glu Asp
        35                  40                  45

Glu Tyr Pro Val Gly Ser Glu Cys Cys Pro Lys Cys Ser Pro Gly
    50                  55                  60
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Tyr Arg Val Lys Glu Ala Cys Gly Glu Leu Thr Gly Thr Val Cys
65 70 75

Glu Pro Cys Pro Pro Gly Thr Tyr Ile Ala His Leu Asn Gly Leu
80 85 90

Ser Lys Cys Leu Gln Cys Gln Met Cys Asp Pro Ala Met Gly Leu
95 100 105

Arg Ala Ser Arg Asn Cys Ser Arg Thr Glu Asn Ala Val Cys Gly
110 115 120

Cys Ser Pro Gly His Phe Cys Ile Val Gln Asp Gly Asp His Cys
125 130 135

Ala Ala Cys Arg Ala Tyr Ala Thr Ser Ser Pro Gly Gln Arg Val
140 145 150

Gln Lys Gly Gly Thr Glu Ser Gln Asp Thr Leu Cys Gln Asn Cys
155 160 165

Pro Pro Gly Thr Phe Ser Pro Asn Gly Thr Leu Glu Glu Cys Gln
170 175 180

His Gln Thr Lys Cys Ser Trp Leu Val Thr Lys Ala Gly Ala Gly
185 190 195

Thr Ser Ser Ser His Trp Val Trp Trp Phe Leu Ser Gly Ser Leu
200 205 210

Val Ile Val Ile Val Cys Ser Thr Val Gly Leu Ile Ile Cys Val
215 220 225

Lys Arg Arg Lys Pro Arg Gly Asp Val Val Lys Val Ile Val Ser
230 235 240

Val Gln Arg Lys Arg Gln Glu Ala Glu Gly Glu Ala Thr Val Ile
245 250 255

Glu Ala Leu Gln Ala Pro Pro Asp Val Thr Thr Val Ala Val Glu
260 265 270

Glu Thr Ile Pro Ser Phe Thr Gly Arg Ser Pro Asn His
275 280 283

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 927 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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BNSDOCID: <WO__9825967A1 I >

CGAGCTGCCG GTCTGAGCCT GAGGCATGGA GCCTCCTGGA GACTGGGGGC 100

CTCCTCCCTG GAGATCCACC CCCAGAACCG ACGTCTTGAG GCTGGTGCTG 150

TATCTCACCT TCCTGGGAGC CCCCTGCTAC GCCCCAGCTC TGCCGTCCTG 200

CAAGGAGGAC GAGTACCCAG TGGGCTCCGA GTGCTGCCCC AAGTGCAGTC 250

CAGGTTATCG TGTGAAGGAG GCCTGCGGGG AGCTGACGGG CACAGTGTGT 300

GAACCCTGCC CTCCAGGCAC CTACATTGCC CACCTCAATG GCCTAAGCAA 350

GTGTCTGCAG TGCCAAATGT GTGACCCAGC CATGGGCCTG CGCGCGAGCC 400

GGAACTGCTC CAGGACAGAG AACGCCGTGT GTGGCTGCAG CCCAGGCCAC 450

TTCTGCATCG TCCAGGACGG GGACCACTGC GCCGCGTGCC GCGCTTACGC 500

CACCTCCAGC CCGGGCCAGA GGGTGCAGAA GGGAGGCACC GAGAGTCAGG 550

ACACCCTGTG TCAGAACTGC CCCCCGGGGA CCTTCTCTCC CAATGGGACC 600

CTGGAGGAAT GTCAGCACCA GACCAAGTGC AGCTGGCTGG TGACGAAGGC 650

CGGAGCTGGG ACCAGCAGCT CCCACTGGGT ATGGTGGTTT CTCTCAGGGA 700

GCCTCGTCAT CGTCATTGTT TGCTCCACAG TTGGCCTAAT CATATGTGTG 750

AAAAGAAGAA AGCCAAGGGG TGATGTAGTC AAGGTGATCG TCTCCGTCCA 800

GCGGAAAAGA CAGGAGGCAG AAGGTGAGGC CACAGTCATT GAGGCCCTGC 850

AGGCCCCTCC GGACGTCACC ACGGTGGCCG TGGAGGAGAC AATACCCTCA 900

TTCACGGGGA GGAGCCCAAA CCACTGA 927

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGTTGAGGCG ACTTTCCCAG GC 22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

BNSDOCID: <WO _ 9825967A1_I_>

AGTTGAGGGG ACTTTCCCAG GC 22

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCTTGATGA CTTGGCCGGA A 21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCTTGATGA CTCAGCCGGA A 21

BNSDOCID: <WO___9825967A1_I_>

WHAT IS CLAIMED IS:

1. Isolated HVEM polypeptide having at least about 80% sequence identity with native sequence HVEM polypeptide comprising amino acid residues 1 to 283 of Figure 1 (SEQ ID NO:1).

2. The HVEM polypeptide of claim 1 wherein said HVEM polypeptide has at least about 90% sequence identity.

3. The HVEM polypeptide of claim 2 wherein said HVEM polypeptide has at least about 95% sequence identity.

4. Isolated native sequence HVEM polypeptide comprising the amino acid sequence of Figure 1 (SEQ ID NO:1).

5. A chimeric molecule comprising the HVEM polypeptide of claim 1 fused to a heterologous amino acid sequence.

6. The chimeric molecule of claim 5 wherein said heterologous amino acid sequence is an epitope tag sequence.

7. The chimeric molecule of claim 5 wherein said heterologous amino acid sequence is an immunoglobulin sequence.

8. The chimeric molecule of claim 7 wherein said immunoglobulin sequence is an IgG.

9. An antibody which specifically binds to the HVEM polypeptide of claim 1.

10. The antibody of claim 9 wherein said antibody is a monoclonal antibody.

11. The antibody of claim 10 which is an agonist antibody.

12. Isolated nucleic acid encoding the HVEM polypeptide of claim 1.

13. The nucleic acid of claim 12 wherein said nucleic acid encodes native sequence HVEM polypeptide comprising amino acid residues 1 to 283 of Figure 1 (SEQ ID NO:1).

14. A vector comprising the nucleic acid of claim 12.

15. The vector of claim 14 operably linked to control sequences recognized by a host cell transformed with the vector.

16. A host cell comprising the vector of claim 14.

17. A process of using a nucleic acid molecule encoding HVEM polypeptide to effect production of HVEM polypeptide comprising culturing the host cell of claim 16.

18. A non-human, transgenic animal which contains cells that express nucleic acid encoding HVEM polypeptide.

19. The animal of claim 18 which is a mouse or rat.

20. A non-human, knockout animal which contains cells having an altered gene encoding HVEM polypeptide.

21. The animal of claim 20 which is a mouse or rat.

22. An article of manufacture, comprising a container and a composition contained within said container, wherein the composition includes HVEM polypeptide or HVEM antibodies.

23. The article of manufacture of claim 22 further comprising instructions for using the HVEM polypeptide or HVEM antibodies *in vivo* or *ex vivo*.

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  1 GCAATGGCGC TGAGTTCCTC TGCTGGAGTT CATCCTGCTA GCTGGGTTCC CGAGCTGCCG GTCTGAGCCT GAGGCATGGA GCCTCCTGGA GACTGGGGGC
    CGTTACCGCG ACTCAAGGAG ACGACCTCAA GTAGGACGAT CGACCCAAGG GCTCGACGGC CAGACTCGGA CTCCGTACCT CGGAGGACCT CTGACCCCCG
  1                                                                                 MetGl uProProGly AspTrpGlyPro

101 CTCCTCCCTG GAGATCCACC CCCAGAACCG ACGTCTTGAG GCTGGTGCTG TATCTCACCT TCCTGGGAGC CCCCTGCTAC GCCCCAGCTC TGCCGTCCTG
    GAGGAGGGAC CTCTAGGTGG GGGTCTTGGC TGCAGAACTC CGACCACGAC ATAGAGTGGA AGGACCCTCG GGGGACGATG CGGGGTCGAG ACGGCAGGAC
 10   ProProTr pArgSerThr ProArgThrA spValLeuAr gLeuValLeu TyrLeuThrP heLeuGlyAl aProCysTyr AlaProAlaL euProSerCys

201 CAAGGAGGAC GAGTACCCAG TGGGCTCCGA GTGCTGCCCC AAGTGCAGTC CAGGTTATCG TGTGAAGGAG GCCTGCGGGG AGCTGACGGG CACAGTGTGT
    GTTCCTCCTG CTCATGGGTC ACCCGAGGCT CACGACGGGG TTCACGTCAG GTCCAATAGC ACACTTCCTC CGGACGCCCC TCGACTGCCC GTGTCACACA
 43  LysGluAsp GluTyrProV alGlySerGl uCysCysPro LysCysSerP roGlyTyrAr gValLysGlu AlaCysGlyG luLeuThrGl yThrValCys

301 GAACCCTGCC CTCCAGGCAC CTACATTGCC CACCTCAATG GCCTAAGCAA GTGTCTGCAG TGCCAAATGT GTGACCCAGC CATGGGCCTG CGCGCGAGCC
    CTTGGGACGG GAGGTCCGTG GATGTAACGG GTGGAGTTAC CGGATTCGTT CACAGACGTC ACGGTTTACA CACTGGGTCG GTACCCGGAC GCGCGCTCGG
 76 GluProCysP roProGlyTh rTyrIleAla HisLeuAsnG lyLeuSerLy sCysLeuGln CysGlnMetC ysAspProAl aMetGlyLeu ArgAlaSerArg

401 GGAACTGCTC CAGGACAGAG AACGCCGTGT GTGGCTGCAG CCCAGGCCAC TTCTGCATCG TCCAGGACGG GGACCACTGC GCCGCGTGCC GCGCTTACGC
    CCTTGACGAG GTCCTGTCTC TTGCGGCACA CACCGACGTC GGGTCCGGTG AAGACGTAGC AGGTCCTGCC CCTGGTGACG CGGCGCACGG CGCGAATGCG
110   AsnCysSe rArgThrGlu AsnAlaValC ysGlyCysSe rProGlyHis PheCysIleV alGlnAspGl yAspHisCys AlaAlaCysA rgAlaTyrAla

501 CACCTCCAGC CCGGGCCAGA GGGTGCAGAA GGGAGGCACC GAGAGTCAGG ACACCCTGTG TCAGAACTGC CCCCCGGGGA CCTTCTCTCC CAATGGGACC
    GTGGAGGTCG GGCCCGGTCT CCCACGTCTT CCCTCCGTGG CTCTCAGTCC TGTGGGACAC AGTCTTGACG GGGGGCCCCT GGAAGAGAGG GTTACCCTGG
143  ThrSerSer ProGlyGlnA rgValGlnLy sGlyGlyThr GluSerGlnA spThrLeuCy sGlnAsnCys ProProGlyT hrPheSerPr oAsnGlyThr

601 CTGGAGGAAT GTCAGCACCA GACCAAGTGC AGCTGGCTGG TGACGAAGGC CGGAGCTGGG ACCAGCAGCT CCCACTGGGT ATGGTGGTTT CTCTCAGGGA
    GACCTCCTTA CAGTCGTGGT CTGGTTCACG TCGACCGACC ACTGCTTCCG GCCTCGACCC TGGTCGTCGA GGGTGACCCA TACCACCAAA GAGAGTCCCT
176 LeuGluGluC ysGlnHisGl nThrLysCys SerTrpLeuV alThrLysAl aGlyAlaGly ThrSerSerS erHisTrpVa lTrpTrpPhe LeuSerGlySer

701 GCCTCGTCAT CGTCATTGTT TGCTCCACAG TTGGCCTAAT CATATGTGTG AAAAGAAGAA AGCCAAGGGG TGATGTAGTC AAGGTGATCG TCTCCGTCCA
    CGGAGCAGTA GCAGTAACAA ACGAGGTGTC AACCGGATTA GTATACACAC TTTTCTTCTT TCGGTTCCCC ACTACATCAG TTCCACTAGC AGAGGCAGGT
210   LeuValIl eValIleVal CysSerThrV alGlyLeuIl eIleCysVal LysArgArgL ysProArgGl yAspValVal LysValIleV alSerValGln

 01 GCGGAAAAGA CAGGAGGCAG AAGGTGAGGC CACAGTCATT GAGGCCCTGC AGGCCCCTCC GGACGTCACC ACGGTGGCCG TGGAGGAGAC AATACCCTCA
    CGCCTTTTCT GTCCTCCGTC TTCCACTCCG GTGTCAGTAA CTCCGGGACG TCCGGGGAGG CCTGCAGTGG TGCCACCGGC ACCTCCTCTG TTATGGGAGT
243  ArgLysArg GlnGluAlaG luGlyGluAl aThrValIle GluAlaLeuG lnAlaProPr oAspValThr ThrValAlaV alGluGluTh rIleProSer

901 TTCACGGGGA GGAGCCCAAA CCACTGA
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276 PheThrGlyA rgSerProAs nHisOP*
```

FIG. 1

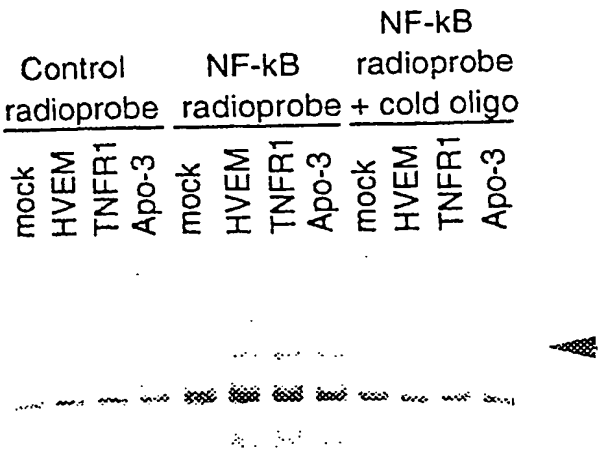


FIG. 2A

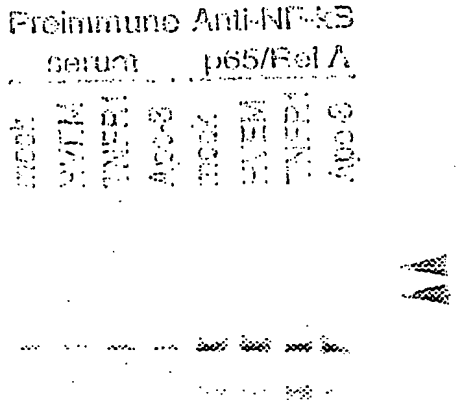


FIG. 2B

BNSDOCID: <WO 9825967A1_I_>

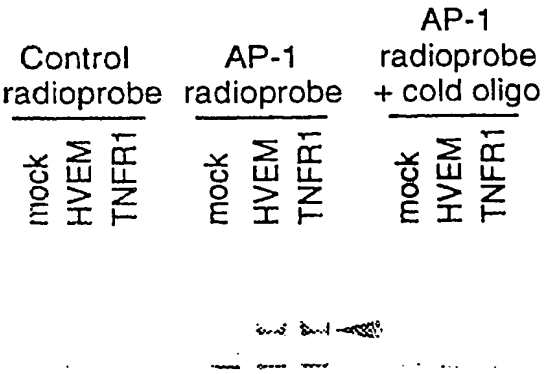


FIG. 3A

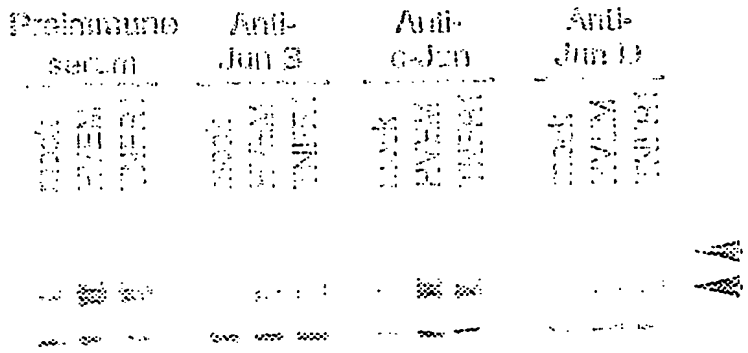


FIG. 3B

BNSDOCID: <WO___9825967A1_I_>

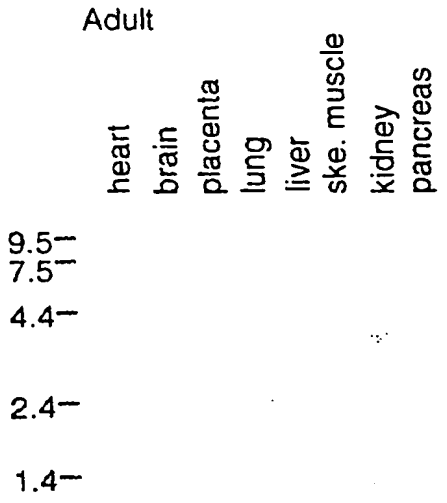


FIG. 4A

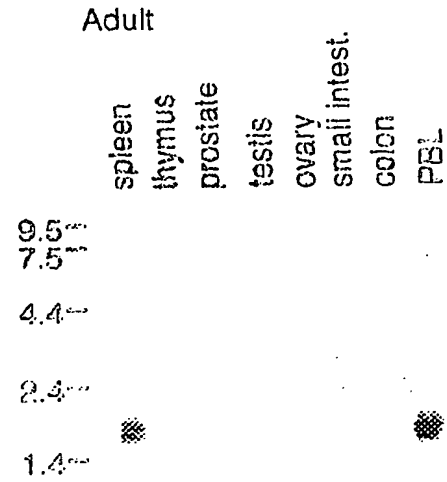


FIG. 4B

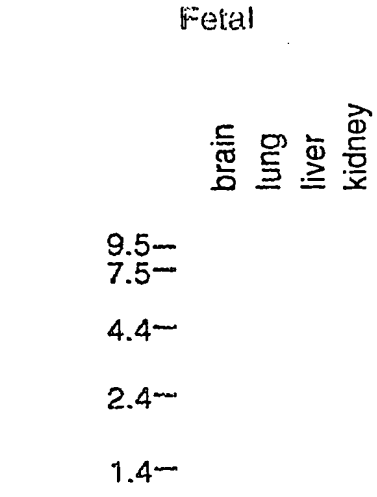


FIG.4C

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INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/US 97/22278

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 96 34095 A (HUMAN GENOME SCIENCES INC) 31 October 1996 see the whole document and specially SEQ.ID.N.2, page 47-48 ---	1-4, 9-17,22, 23 5-8
Y	WO 96 03141 A (HOFFMANN LA ROCHE) 8 February 1996 see abstract see page 3, line 21 - page 6, line 27 --- -/--	5-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 April 1998	15. 05. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R.I. MONTGOMERY ET AL., : "Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family" CELL, vol. 87, 1996, CAMBRIDGE, MA, US, pages 427-436, XP002062424 cited in the application see the whole document	1-4, 12-17
A	US 5 447 851 A (BEUTLER BRUCE A ET AL) 5 September 1995 see the whole document	5-8
P,X	WO 97 04658 A (UNIV NORTHWESTERN) 13 February 1997 see the whole document and specially Fig 2	1-4,9-17
P,X	B.S. KWON ET AL., : "A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, 30 May 1997, BETHESDA, MD, US, pages 14272-14276, XP002062425 see the whole document	1-4,12, 13
T	K.B. TAN ET AL., : "Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells" GENE, vol. 204, 19 December 1997, AMSTERDAM, NL, pages 35-46, XP004100692 see the whole document	1-4,12, 13

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/22278

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WO 9634095 A	31-10-96	AU 2363695 A	18-11-96
		EP 0822984 A	11-02-98
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		PL 318501 A	23-06-97
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WO 9704658 A	13-02-97	NONE	

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